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Transient Cell Cycle Arrest of *Saccharomyces cerevisiae* by Amino Acid Analog β-2-DL-Thienylalanine

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When treated with the amino acid analog β-2-DL-thienylalanine, cells of the yeast *Saccharomyces cerevisiae* accumulated in the G1 portion of the cell cycle at the "start" event. This G1 arrest was accompanied by a rapid decrease in the rate of labeling of ribonucleic acid (RNA) with little effect on the rate of labeling of protein. When we examined which aspect of RNA metabolism was most affected by β-2-DL-thienylalanine treatment, we found a dramatic decrease in the production of ribosomal precursor RNA. These results are consistent with previous findings which show a correlation between G1 arrest and reduced ribosomal precursor RNA production. The G1 arrest brought about by β-2-DL-thienylalanine was transient; cells remain arrested in G1 only for several hours. Release from G1 arrest appeared to be accompanied either by metabolism or sequestration of the analog.

Regulation of cell division in the yeast *Saccharomyces cerevisiae*, like most eucaryotes, occurs within the G1 portion of the cell cycle. Hartwell (7) has defined a period within G1 referred to as "start" which must be completed to initiate a new cell cycle. Starvation for any one of many required nutrients arrests cells at the "start" point of the cell cycle.

Studies of the mechanism of cell cycle control have been facilitated by compounds which cause specific G1 arrest. Among compounds leading to G1 arrest are the zinc-chelating agent *o*-phenanthroline (10) and the amino acid analog L-ethionine (14). These agents also lead to a decrease in rRNA production, with little effect on general protein synthesis. These observations have led us to propose that some aspect of transcription of rRNA genes is involved in the regulation of cell division (10, 14).

One means of assessing this proposal is to determine whether similar effects on transcription of rRNA genes are found for apparently unrelated compounds showing cell cycle activity. To this end we have screened a large number of analogs for uniform cell cycle effects. Of these, one compound, β-2-DL-thienylalanine (TA), caused cells to arrest transiently in G1. Here we describe the macromolecular and cell cycle effects of this phenylalanine analog.

MATERIALS AND METHODS

Strains and medium. The diploid strain AG1-7 (*ural his6*) and its isogenic haploid segregant GR2 (a *ural his6*) have been described elsewhere (10, 11). Cells were grown in a liquid synthetic medium (YNB) (9) supplemented with uracil (20 μg/ml) and histidine (40 μg/ml). Radioactively labeled uracil and histidine were obtained from New England Nuclear Corp. TA was obtained from Sigma Chemical Co. The mating pheromone α-factor was prepared by the method of Bücking-Throm et al. (2). Determination of cell number and proportion of cells in G1 has been described elsewhere (6).

Determination of macromolecular metabolism. To measure the incorporation of labeled precursors into macromolecules and to determine the degradation of RNA, we employed methods previously described (11). Briefly, synthesis of macromolecules was measured by incorporation of radioactive precursors into trichloroacetic acid-precipitable material. Degradation of RNA was measured by quantitating release of acid-soluble radioactivity into trichloroacetic acid-extractable pools.

For RNA fractionation, cells were frozen in Dry Ice-ethanol, resuspended in 10^{-2} M Tris·hydrochloride (pH 7.5) containing 10^{-3} M EDTA, and lysed with a French pressure cell. The RNA was extracted and resolved by electrophoresis through 2.8 or 10% polyacrylamide gels (10). Gels were processed for scintillation counting as described previously (11).

UTP and CTP pools were determined as follows. Cells of strain AG1-7 were labeled for at least six generations with [^{14}C]uracil. After 1 and 4 h in the presence of TA (100 μg/ml), samples were labeled for 5 min with [^{3}H]uracil. Nucleoside triphosphate pools were extracted with 1 M formic acid overnight at 4°C and resolved by chromatography on polyethyleneimine plates as described (3). Spots of UTP and CTP comigrating with the extracts were scraped off the plates, hydrated with water, and counted in Aquasol.

RESULTS

Cell cycle effects of TA. In the budding yeast, the onset of S phase is correlated with the

production of a bud; a cell without a bud is in G1 (7). When cells of the diploid strain AG1-7 were treated with TA, the cell number increased 1.8-fold, and after 8 h in the presence of TA the proportion of cells in G1 had increased to greater than 90% (Fig. 1B). No further increase in cell number occurred during the next 12-h period. Finally, 17 h after the addition of TA, the proportion of cells without buds fell rapidly, and the cell number began to increase some 3 h later (data not shown). The duration of this transient G1 arrest was proportional to the initial TA concentration employed and inversely proportional to the cell concentration at the time of TA addition. A 17-h arrest period was routinely found with TA at 100 μg/ml with an initial cell concentration of 5×10^6 cells per ml. Unless otherwise specified, TA additions were to this final concentration.

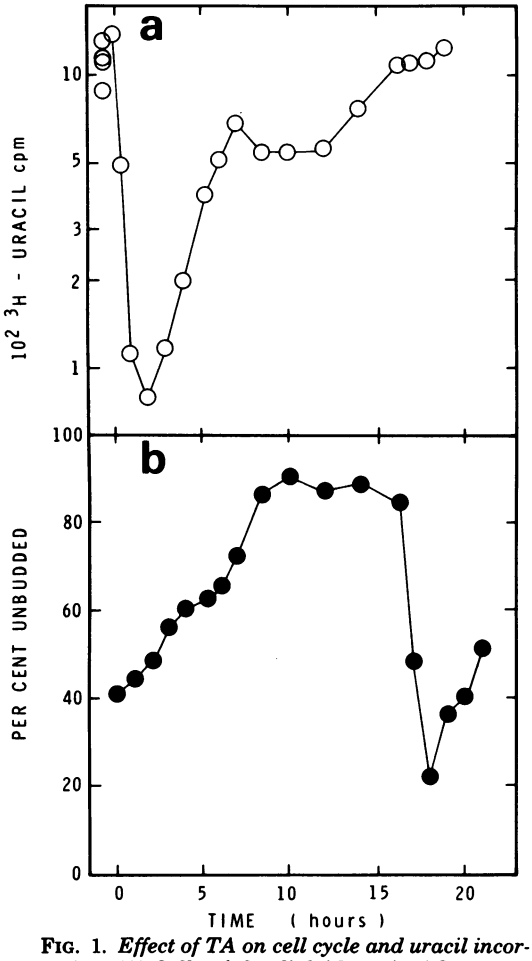

FIG. 1. *Effect of TA on cell cycle and uracil incorporation. (A) Cells of the diploid strain AG1-7 were treated with TA (100 μg/ml), and at intervals portions were removed and incubated for a further 5 min in the presence of [^{3}H]uracil (10 μCi/ml) before addition of trichloroacetic acid. (B) After treatment with TA (100 μg/ml), samples were removed for determination of the proportion of cells without buds. In the budding yeast, the onset of DNA synthesis is coincident with bud emergence; thus, cells without buds are in the G1 portion of the cell cycle (7).*

During the period of G1 arrest, cells continued to increase in size. Cell size distributions were determined with a Coulter Channelizer as described previously (9). Median cell volume increased up to fourfold over normal values for growing cells. Upon resumption of cell division, the size distribution of cells returned to that of an untreated population (data not shown).

To determine the position in the cell cycle at which TA-treated cells were blocked, we used a technique referred to as order-of-function mapping. This method of analysis has been used to order, in a linear sequence, various cell cycle blocks in yeast (8). Haploid cells of **a** mating type arrest in G1 at the regulatory point referred to as start when treated with the mating pheromone α-factor (2). In one experiment, cells of the isogenic **a** haploid strain GR2 were treated with α-factor. Three hours later, cells were collected and resuspended in fresh medium with or without TA (100 μg/ml). In the absence of α-factor or TA, cell division (as determined by the appearance of buds) resumed within 1.5 h (Fig. 2A). In the presence of TA, cells previously arrested in G1 by α-factor remained in G1. Thus, cells arrested by α-factor were blocked at or before the TA-sensitive step. The reciprocal shift experiment was also performed. Cells arrested in G1 by exposure to TA for 7 h were collected and resuspended in fresh medium with or without α-factor. In the absence of TA or α-factor, cells resumed progress through the cell division cycle within 1 h. Cells arrested in G1 by TA treatment remained in G1 in the presence of α-factor (Fig. 2B). Thus, cells arrested by TA were at or before the α-factor-sensitive step. Taken together, these experiments show the α-factor-sensitive step (start) and the TA-sensitive step are interdependent and function at the same point in the cell cycle.

Metabolism of TA. Since TA-induced G1 arrest is transient, cells may remove TA from the medium, or alternatively may become resistant to the effects of TA. Cells of strain AG1-7 which had just initiated the first division cycle after TA-induced arrest were subjected to further addition of TA. These cells completed the division cycle and arrested in the next G1 period. Thus, cells overcoming the TA-induced G1 arrest were still sensitive to further TA treatment. To determine whether cell cycle-active agents remained in the medium of cells overcoming TA-induced arrest, spent medium was obtained

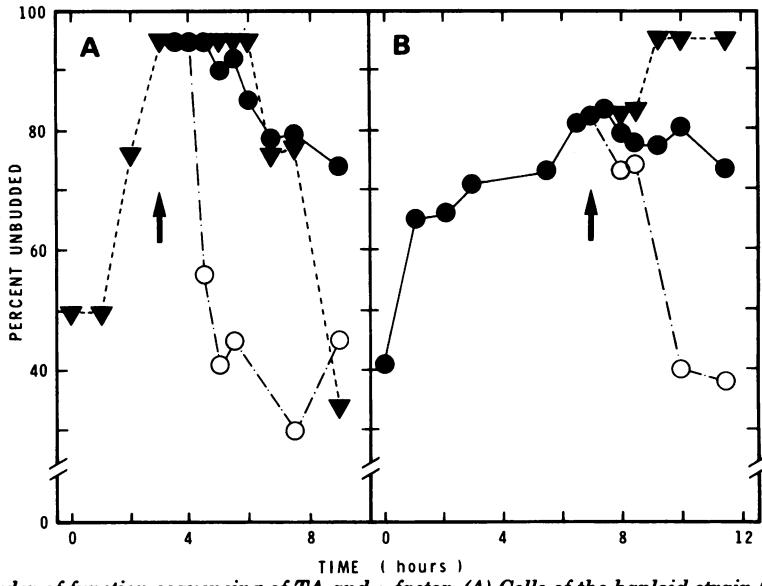

FIG. 2. *Order-of-function sequencing of TA and α-factor. (A) Cells of the haploid strain GR2 were treated with α-factor at zero time. After 3 h (at the arrow), some of the cells were collected and resuspended in fresh medium with or without TA (100 μg/ml). At indicated times samples were removed for morphological assessment. (B) Cells of strain GR2 were treated at zero time with TA (100 μg/ml), then collected and resuspended after 7 h (at the arrow) in fresh medium with or without α-factor. Symbols: ○, cells in medium alone; ▼, cells in medium containing α-factor; ●, cells in medium containing TA (100 μg/ml).*

from a culture of strain AG1-7 in which cells were initiating the first cell cycle after spontaneous recovery from TA-induced G1 arrest. Cells not previously exposed to TA displayed no cell cycle effects when placed in this spent medium. Thus, recovery from this G1 arrest is accompanied by loss of cell cycle-active agents from the medium.

Effect of TA on RNA and protein synthesis. Yeast cells when treated with the G1-arresting compounds, *o*-phenanthroline (10) or L-ethionine (14), show dramatic decreases in the rates of rRNA synthesis, whereas the rates of protein synthesis remain relatively unaffected. We determined whether transient cell cycle arrest due to exposure of cells to TA was also associated with a decrease in the rate of RNA production. As shown in Fig. 3, the immediate effect of the addition of TA was a rapid decrease in the rate of [^{3}H]uracil incorporation into acid-precipitable material. This diminished rate of exogenous uracil incorporation by the uracil-auxotrophic strain AG1-7 was not solely a reflection of changes in the specific activities of the UTP and CTP pools. This conclusion was supported by two types of experiments. First, we examined the effect of TA addition on turnover of macromolecules. Cells were grown for several generations in medium containing [^{14}C]uracil, washed free of labeled precursor, and incubated in unlabeled medium for an additional 2 h before TA treatment. The appearance of acid-soluble label from TA-treated cells was less than 1% up to 6 h after the analog was added (data not shown). Second, examination of the relative specific activities of UTP and CTP pools after the addition of TA did indeed indicate changes in pool specific activities; however, these changes were insufficient to account for the greater decrease in the rate of exogenous uracil incorporation (Table 1). Therefore, exposure of cells to TA was associated with decreased rates of RNA production.

Previous work (10, 14) has suggested that the production of ribosomal precursor RNA is differentially affected when cells are treated with compounds leading to G1 arrest. To examine the effect of TA treatment on the rate of production of different RNA species, a portion of a culture of cells prelabeled by growth for several generations with [^{14}C]uracil was treated with TA. Sixty minutes after TA addition, portions of the treated and control cultures were incubated with [^{3}H]uracil; after 2, 4, and 6 min of incubation, cells were harvested, and the RNA was extracted and resolved by either 2.8% (Fig. 4A) or 10% polyacrylamide gel electrophoresis as described elsewhere (14). Newly synthesized RNA was quantitated by normalizing the amount of ^{3}H incorporated into various RNA species to the amount of ^{14}C recovered in 25S rRNA or in tRNA. After correction for altered pool specific

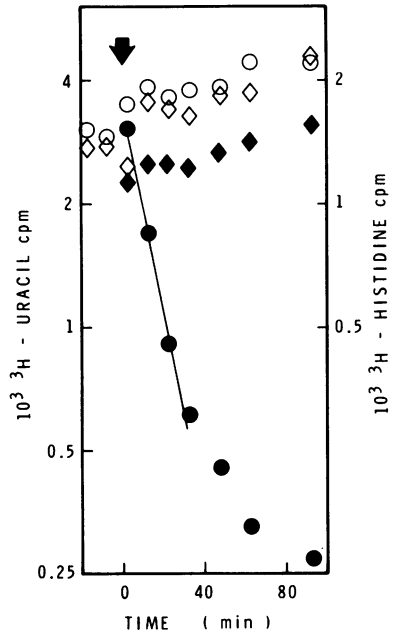

FIG. 3. *Immediate effect of TA addition on uracil and histidine incorporation. At zero time (indicated by the arrow), a growing culture of cells of strain AG1-7 was divided, and half was treated with TA (100 μg/ml). At intervals, portions were removed and incubated for 5-min periods with either [^{3}H]uracil (10 μCi/ml) or [^{3}H]histidine (10 μCi/ml) at intervals after TA addition (100 μg/ml). Symbols: ●, [^{3}H]uracil incorporation in the presence of TA; ○, [^{3}H]uracil incorporation in the absence of TA; ◆, [^{3}H]histidine incorporation in the presence of TA; ◇, [^{3}H]histidine incorporation in the absence of TA.*

activities, the data showed no effect of TA addition on the rate of production of 4S RNA (data not shown). However, the rate of production of 35S ribosomal precursor RNA decreased about fivefold (Fig. 4B). Thus, the phenylalanine analog TA has both molecular and cell cycle effects similar to those of the apparently unrelated compound ethionine.

During the period of transient G1 arrest, the rate of labeling of RNA gradually returned to initial levels (Fig. 1A). If the period of transient arrest was altered by changing the TA concentration or the cell concentration as described above, the time required for recovery of the rate of RNA labeling was correspondingly altered. Regardless of the duration of the G1 arrest period, the rate of RNA labeling returned to initial rates before the resumption of bud initiation and cell division.

The rate of [^{3}H]histidine incorporation into acid-precipitable material remained near control levels during treatment with TA. After prolonged periods of TA-mediated G1 arrest, we consistently saw a decrease in the rate of histidine incorporation, as yet unexplained.

DISCUSSION

Three apparently unrelated compounds, when added to growing yeast cells, cause G1 arrest at start. These compounds include the zinc-chelating agent *o*-phenanthroline (10), the methionine analog L-ethionine (14), and the phenylalanine analog TA. In the case of TA treatment, the G1 arrest was transient, most likely because of metabolism or sequestration of the analog.

Earlier work from this laboratory (10, 14) has suggested that decreased ribosomal precursor RNA production may be involved in regulation of cell division in yeast. The effects of TA treatment described here are consistent with this hypothesis. General RNA synthesis as assessed by label incorporation rates was decreased; by gel electrophoretic analysis the bulk of this decrease was ascribed to decreased ribosomal precursor RNA production. Protein synthesis, also determined by labeled precursor incorporation, was relatively unaffected by TA treatment. This observation further suggests that mRNA production was not significantly decreased. The correlation between G1 arrest and decreased ribosomal precursor RNA production is found for treatment with all three apparently unrelated compounds. This correlation greatly strengthens the suggestion that rRNA production may be involved in cell cycle regulation.

Amino acid analogs have been used as inhibitors of growth in both procaryotes (13, 17) and eucaryotes (1, 4, 5, 12, 15, 16, 18). Studies with eucaryotic cells have also suggested that amino acid analogs may have effects on the cell cycle (1, 12, 15, 16, 18). Analogs of phenylalanine affect both mitosis and the structure of the mitotic spindle (16) in mammalian cells. For example, many analogs of phenylalanine, including TA, blocked the progress of HeLa cells from G2 to mitosis (19). In contrast to this general cell cycle effect shown by phenyalanine analogs in animal cells, only TA gives cell cycle effects in yeast. Of other phenylalanine analogs tested, only *o*-fluorophenylalanine inhibited growth of *S. cerevisiae*, but did not lead to uniform cell cycle effects (unpublished data).

TABLE 1. *Nucleoside triphosphate pools during TA treatment*

Nucleoside triphosphate	$^3H/^{14}C$ ratio[a]		
	−TA	+TA (1 h)	+TA (4 h)
UTP	1.00	0.57	0.69
CTP	0.98	0.38	0.37

[a] Values shown are $^3H/^{14}C$ ratios normalized to UTP ratios in untreated cultures.

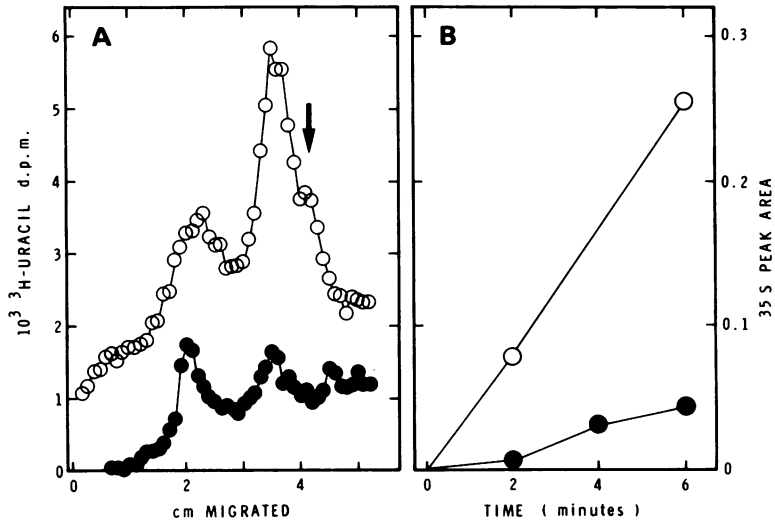

FIG. 4. *Appearance of labeled 35S RNA during TA treatment. (A) A growing culture of cells of strain AG1-7 was prelabeled with [^{14}C]uracil for six generations and then divided. Half of the culture received TA to 100 μg/ml. One hour later, portions of the TA-treated and control cultures were incubated for 6 min with [^{3}H]-uracil. Two separate gel profiles are shown, aligned to the ^{14}C-labeled 25S rRNA (arrow). Under these conditions, the 35S material migrated approximately 2 cm. Symbols: ○, ^{3}H-labeled RNA from control cells; ●, ^{3}H-labeled RNA from TA-treated cells. The radioactivity values from the TA-treated samples are doubled for clarity. (B) ^{3}H radioactivity in the 35S peak after 2-, 4-, and 6-min incubation periods was normalized to ^{14}C recovery in the 25S rRNA peak. These values were then corrected for pool size alterations (Table 1) and for specific activity of [^{3}H]uracil used during each incubation period. Symbols: ●, normalized ^{3}H in 35S material from TA-treated cells; ○, normalized ^{3}H in 35S material from control cells.*

As suggested by others, TA is in some aspects structurally similar to methionine (5). However, two pieces of evidence make it unlikely that TA causes similar cell cycle and macromolecular effects as ethionine because of these structural similarities. In rats the toxicity of TA could be reversed by phenylalanine, but not by methionine (5). For *S. cerevisiae* it has been demonstrated that inhibition of growth by TA could not be abrogated by addition of a number of amino acids, including methionine (5). We have also noted that the addition, along with TA, of a twofold-greater molar concentration of L-methionine had no effect on cell kinetics of G1 arrest, and a ninefold excess only reduced the transient arrest period somewhat; in contrast, a molar concentration of L-phenylalanine no more than 80% that of TA completely abolished the G1 arrest response. Thus, TA is probably not functioning metabolically as another methionine analog as is ethionine.

Cell cycle arrest in yeast can therefore be brought about by some apparently unrelated amino acid analogs. That uniform cell cycle arrest is not a general feature of analog-mediated inhibition of growth is shown by effects of other methionine (14) and phenylalanine (unpublished data) analogs. Results concerning ethionine metabolism in yeast cells suggest that at G1-arresting concentrations this analog is not incorporated into protein, and does not inhibit tRNA charging or the process of protein synthesis (for discussion, see reference 14). Although no similar studies on TA metabolism have been reported, we found that TA had little effect on amino acid incorporation. For these reasons, it is likely that these G1-arresting agents are not affecting cell division regulation through the process of protein synthesis.

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